Video Article

Long-term Silencing of Intersectin-1s in Mouse Lungs by Repeated Delivery of a Specific siRNA via Cationic Liposomes. Evaluation of Knockdown Effects by Electron Microscopy

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Abstract

Previous studies showed that knockdown of ITSN-1s (KD_{ITSN}), an endocytic protein involved in regulating lung vascular permeability and endothelial cells (ECs) survival, induced apoptotic cell death, a major obstacle in developing a cell culture system with prolonged ITSN-1s inhibition[1]. Using cationic liposomes as carriers, we explored the silencing of ITSN-1s gene in mouse lungs by systemic administration of siRNA targeting ITSN-1 gene ($siRNA_{ITSN}$). Cationic liposomes offer several advantages for siRNA delivery: safe with repeated dosing, nonimmunogenic, nontoxic, and easy to produce[2]. Liposomes performance and biological activity depend on their size, charge, lipid composition, stability, dose and route of administration[3]Here, efficient and specific KD_{ITSN} in mouse lungs has been obtained using a cholesterol and dimethyl dioctadecyl ammonium bromide combination. Intravenous delivery of $siRNA_{ITSN}$/cationic liposome complexes transiently knocked down ITSN-1s protein and mRNA in mouse lungs at day 3, which recovered after additional 3 days. Taking advantage of the cationic liposomes as a repeatable safe carrier, the study extended for 24 days. Thus, retro-orbital treatment with freshly generated complexes was administered every 3rd day, inducing sustained KD_{ITSN} throughout the study[4]. Mouse tissues collected at several time points post-$siRNA_{ITSN}$ were subjected to electron microscopy (EM) analyses to evaluate the effects of chronic KD_{ITSN}, in lung endothelium. High-resolution EM imaging allowed us to evaluate the morphological changes caused by KDITSN in the lung vascular bed (*i.e.* disruption of the endothelial barrier, decreased number of caveolae and upregulation of alternative transport pathways), characteristics non-detectable by light microscopy. Overall these findings established an important role of ITSN-1s in the ECs function and lung homeostasis, while illustrating the effectiveness of siRNA-liposomes delivery *in vivo*.

Video Link

The video component of this article can be found at https://www.jove.com/video/50316/

Introduction

Naked siRNA cannot penetrate the cell membrane, being negatively charged, and it is easily degraded by enzymes in blood, tissues, and cells. Even with recently structural modifications to improve stability, siRNA accumulation at the target site after administration is extremely low and requires an efficient intracellular vehicle[5]. Cationic liposomes emerged as safe nucleic acids carriers with the potential to transfer large pieces of DNA/RNA into cells by encapsulating and protecting the nucleic acids from enzymatic degradation[6]. Also, cationic liposomes spontaneously interact with DNA/RNA, thus promoting gene transfer to the cells[2]. More recently liposomes have been applied to deliver vaccines and low-molecular-weight drugs[7]. Liposomal delivery of microRNA-7-expressing plasmid overcomes epidermal growth factor receptor tyrosine kinase inhibitor-resistance in lung cancer cells[8]. When targeting the vascular endothelium, the intravenous delivery is essential because the complexes are unlikely to cross the endothelial barrier and extravasate in to the interstitium[9]. By comparison to other organs, ECs from the microvasculature of the lung have the greatest uptake and avidly internalize cationic liposome and DNA/RNA complexes, followed by the lymph nodes and Peyer's patches[9]. Intravenous delivery in rodent models of siRNA via retro-orbital or tail vein injections has been proven harmless even in high concentrations, such as 50 mg/kg[10]. In the published literature the composition of cationic liposomes differs, based on the lipids formulation and their equimolar ratio[11, 12]. There is a wide array of potential applications using cationic liposomes for gene delivery *in vivo*, whether targeting down-regulation/over-expression of the proteins, delivery of vaccines or anti-tumor therapies[13-15]. Important to remember is that the efficiency of DNA/RNA-cellular membrane interaction is regulated by the shape coupling between generated complexes and membrane lipids. This suggests that tailoring the lipids composition to the targeted cellular membrane profiles may be beneficial and result in high rates of transfection in a particular cell type[6].

Protocol

1. Cationic Liposomes Preparation

1. Autoclave a clean round-bottom flask to be used for lipososmes preparation.
2. Prepare stock solutions:
   a. - Dissolve 200 mg of dimethyl dioctadecyl ammonium bromide (DOAB) in 10 ml chloroform using a small glass bottle.
   b. - Dissolve 200 mg of cholesterol in 10 ml chloroform using a small glass bottle.
   c. - Prepare and autoclave to sterilize 5% glucose in 100 ml of RNA/DNA-ase free distilled water.
3. Rinse the round-bottom flask with chloroform. Add 5-10 ml to the flask, swirl, and let it sit for few minutes. Keep the flask covered with aluminum foil at all times. Empty the chloroform from the flask.
4. Preparation of liposomes:
   a. - Add 315 µl of DOAB stock solution to the round-bottom flask.
   b. - Add 200 µl of cholesterol stock solution to the flask.
   c. - Add 9.5 ml of chloroform to the flask and mix by swirl.
5. Set the rotavapor at 37 °C, 100 rotations per minute (rpm) or more (100-120 rpm). The rotavapor should be connected to a water-pump vacuum control with a suction tube properly attached.
6. Connect the argon gas tank and the rotavapor.
7. Attach the flask to the rotavapor and adjust the optimal settings before lowering it in the water bath. Open the first valve for the argon completely by turning it counterclockwise. Always open and close this valve first. The second valve controls the argon pressure blowing in to the flask. The pressure should be always gentle.
8. Lower the rotavapor in the water bath, enough for the flask to touch the water without being completely submerged and turn on the speed.
9. Wait until all the chloroform evaporates, about 10 min. Then stop the machine.
10. Turn off the rotavapor speed and the argon tank valves. Now the flask should be removed.
11. Add 2 ml of 5% glucose to the flask to dissolve the lipids.
12. Scratch the bottom of the flask thoroughly using 1 ml pipette tip to dissolve all lipids. After scratching, remove any remaining lipid from the 1 ml tip into the flask, if necessary, with a second tip.
13. Incubate the obtained solution in 42 °C water bath while slowly vortexing (15 rpm) the flask.
14. Fill the sonicator vat with cold water. Sonicate the solution for at least 20-30 min with the flask held up and the bottom just barely touching the water.
15. Heat the flask in the rotavapor's water bath for 20 min at 42 °C, at zero speed, with the round bottom immersed in water, this time.
16. Filter the liposomes through a 0.47 micron and subsequently, 0.22 micron syringe filters.
17. Using a small extruder, filter the liposomes through a 50 nm membrane to generate a homogenous population of unilamellar liposome vesicles.
18. Transfer the liposomes to an Eppendorf tube and place it on ice.

2. Prepare the Liposomes: siRNA-ITSN-1 Complexes

1. Resuspend the on-target mouse $siRNA_{ITSN}$ in siRNA buffer containing 20 mM HEPES and 150mM sodium chloride, pH 7.4, to a final concentration of 2 µg/µl. Keep it on ice before mixing.
2. Prepare the liposomes:$siRNA_{ITSN}$ complexes at a ratio of 8 nmol liposomes:2 µg RNA (or 400 nmol liposomes:100 µg $siRNA_{ITSN}$). In the present study, we added 50 µl of $siRNA_{ITSN}$ from the stock solution (50 µM) to 100 µl of freshly prepared liposomes using a RNA/DNA-ase free Eppendorf tube. Note that the concentration of $siRNA_{ITSN}$ is very important because a maximum of 150 µl of mixture can be bilaterally and retro-orbital injected in the mouse at one time.
3. Keep the mixture on ice while waiting to inject the mice.

3. Mouse Retro-orbital Vein Injection (Internal Angle of the Eye Socket)

All mouse experiments were approved and performed in accordance with the guidelines of Rush University Institutional Animal Care and Use Committee.

1. Anesthetize the mouse by intra-peritoneal injection of a 50 mg/kg body weight ketamine and 5 mg/kg body weight xylazine (commercially available mixture). Depending on the animal weight and age, adjust the anesthetics volume and concentration. The mice used in the study mice are CD1 male mice, 6-8 weeks old and weigh around 25 grams. The syringes are tuberculin 1 ml type with the needle attached.
2. Hold mouse ears with one hand and turn the mouse down on a side to expose the internal angle of the eye. Aim medial to the plica semilunaris; avoid touching the eyeball.
3. Approach the lacrimal caruncle of the eye holding the syringe containing the mixture at a 45 degrees angle in all three axes.
4. Slowly inject the mixture and let the mouse to recover with the injected side up. If a large liquid droplet forms at the needle insertion place, retract the needle, suction back the droplet into the syringe and try again. Repeated injections are best done by alternating the eyes, to allow perfect recovery. If unsure, this technique can be checked by injecting blue dye (50 µl, 0.5% crystal violet in methanol) and exposing the mouse lungs to observe the blue vasculature.
5. The mice in this study were injected every 3rd day for 3 weeks without fatalities or adverse effects.

4. Mouse Lung Perfusion and Tissue Collection for Biochemical Studies

0. Set the peristaltic pump to run at 1.5 ml/min.
1. Prepare the setting: ventilator, operating table, instruments, sterile cotton swabs, q-tips, strings, beakers, distilled water, Hank's balanced salt, tracer.
2. Anesthetize the mouse by intraperitoneal injection of the anesthetic mixture (100 mg/kg body weight ketamine and 10 mg/kg body weight xylazine).
3. At the neck level, remove the salivary glands and expose the trachea.
4. Start with laparotomy, excise the diaphragm, and follow with thoracotomy, exposing the lungs and the heart.
5. Remove the thymus.
6. Using the light microscopy for better accuracy, catheterize the pulmonary artery.
7. Do tracheostomy and intubate the mouse using the tracheostoma. Set the ventilator to a rate of 150 strokes/min and stroke volume of 150 µl.
8. As outlet, open the left atrium.
9. Perfuse the mouse lung vasculature free of blood for 5 min, using the peristaltic pump and Hank's solution warmed at 37 °C.
10. Perfuse the tracer (8 nm gold-albumin[16]) for additional 10 min at the same flow rate.
11. Flush the unbound tracer by 5 min lung perfusion with Hank's solution.
12. Follow with *in situ* fixation of the lungs by 10 min perfusion of 4% (wt/vol)formaldehyde, 2.5% glutaraldehyde, and 1% tannic acid in 0.1 PIPES buffer, pH 7.2.
13. Collect the lungs, remove the excessive tissue, cut small blocks (3/3 mm), and place them in to a labeled scintillation vial containing 2 ml of fixative mixture.

The mice will expire fully anesthetized during the procedure.

5. Mouse Lung Tissue Processing for EM

1. Prepare stock solutions: 1% Palade OsO_4, acetate veronal, and Kellenberger buffer.
   a. - 1% Palade-OsO_4 buffer contains: 1 ml acetate veronal stock, 1.25 ml 4% OsO_4, 1 ml of 0.1 N hydrochloric acid, and distilled water up to 5 ml of final volume.
   b. - Acetate veronal stock solution is obtained by dissolving 1.15 g sodium acetate andydrous and 2.94 g barbital in 100 ml of distilled water.
   c. -Kellenberger solution contains 2 ml of acetate veronal stock, 2.8 ml of 0.1 N hydrochloric acid, 0.05 g uranyl acetate, and 5.1 ml of distilled water, pH= 6.
2. Wash the lung blocks in 0.1 M sodium cacodylate buffer, pH= 7.4, briefly 3 times.
3. Fix the specimens in the 4% (wt/vol) formaldehyde, 2.5% glutaraldehyde in 0.1 PIPES buffer, pH 7.2, for 1 hr, at room temperature.
4. Post-fix with 1% Palade-Osmium for 1 hr in the hood, on ice, in the dark.
5. Rinse once with Kellenberger buffer.
6. Incubate specimens in Kellenberger buffer for 2 hr to overnight, at room temperature.
7. Rinse once in 50% ethanol.
8. Dehydrate with graded series of ethanol, 5 min/each: 70%, 95%, then 2 x 15 min 100% ethanol.
9. Switch to 100% propylene oxide, 2 x 15 min.
10. Remove the propylene oxide and add a mixture of 50% propylene oxide and 50% Epon 812, incubate overnight, rotating on a wheel, at room temperature.
11. Next morning, remove the mixture and add fresh Epon 812, at least for 4-5 hr on the wheel.
12. Using doubled tapered molds, filled half way with fresh 100% Epon 812, add selected specimens and place them to the edges.
13. Move the molds to the incubator set at 60 °C and let them cure for 48-72 hr.
14. When polymerized, send the blocks to be thin (60-70 nm thick) sectioned.

Representative Results

ITSN-1s protein and mRNA levels were monitored at several time points after $siRNA_{ITSN}$ delivery by Western blot and conventional and quantitative PCR as in[4]. ITSN-1s protein and mRNA levels in $siRNA_{ITSN}$-treated mouse lungs were about 75% lower by reference to controls during continuous knockdown of ITSN-1s for 21 days. Without ITSN-1s, dynamin-2, a major interacting partner of ITSN-1s and essential player in detachment of caveolae from the plasma membrane is not efficiently recruited to the endocytic site and thereby, caveolae detachment from the plasma membrane and formation of free vesicular carriers is disrupted[1, 17]. Therefore, ineffective membrane fission and impaired formation of free vesicular carriers caused deficient endocytosis and transendothelial transport, disruption of inter-endothelial barrier and pulmonary edema (**Figure 1**). Moreover, down-regulation of ITSN-1s up regulated alternative transport pathways to compensate for deficient endocytosis. We noticed membranous rings (**Figures 2A** and **2a1**), pleomorphic tubules (**Figure 2B**), and enlarged endosomes fused with typical caveolae (**Figure 2C**) actively involved in the uptake and transport gold-albumin. A significant finding is the decrease in caveolae number in ITSN-1s deficient mouse lung endothelium by reference to controls, **Table 1**. Prolonged ITSN-1s inhibition for 24 days by repeated delivery of cationic liposomes/$siRNA_{ITSN}$ reduced the pulmonary edema in mice by partially restoring the inter-endothelial barrier integrity. EM morphological studies showed that the 8nm gold-albumin tracer could not penetrate the inter-endothelial junctions, at this time point. Instead, they formed filtration residues in the luminal introit of the junction (**Figure 3**). However, the perivascular spaces showed some dilation and mild edema, suggesting that junctions are impermeable to this size of the particles, but still leaky. Also, the morphological intermediates of alternative endocytic pathways are active and present in higher numbers. Noticeably, the morphometric analyses indicated that the number of membranous ring/tubules labeled by gold-albumin particles increased by 14 fold in ITSN-1s chronic deficient mouse lung endothelium when compared to controls, **Table 1**.

Caveolae number is partially restored, only 17.8% decrease, by reference to controls. Thus, our results demonstrate that repeated delivery of cationic liposomes/siRNA$_{ITSN}$ complexes is a suitable methodology to study the effects of long-term protein knockdown *in vivo*.

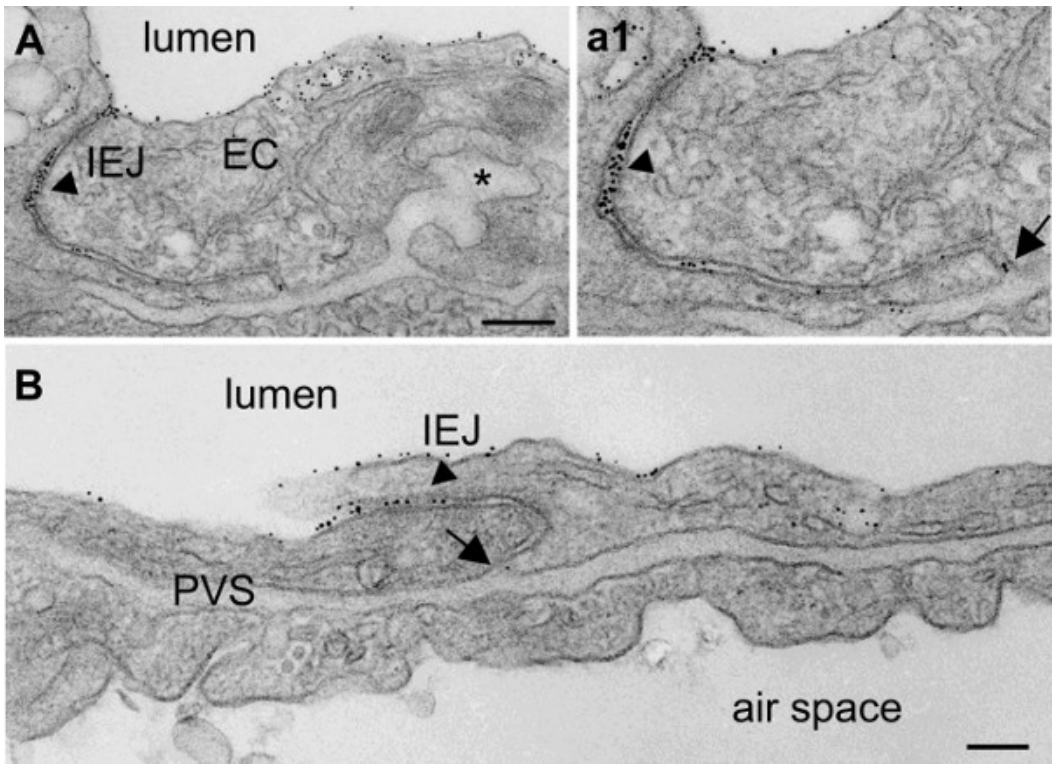

Figure 1. Representative electron micrographs showing open interendothelial junctions (IEJs) labeled throughout their length by 8 nm gold-albumin particles. Arrowhead in **A** and magnified **a1**, points to three-four gold-albumin particles located close to each other in the same plan, indicative of the wide opening of the IEJ. Gold particles are also associated with the abluminal exit of IEJs (**A, B** - arrows). Note also the limited number of caveolae and dilation of the pericapillary space pcs; asterisks). Bars: 200 nm (A); 100 nm (B, a1).

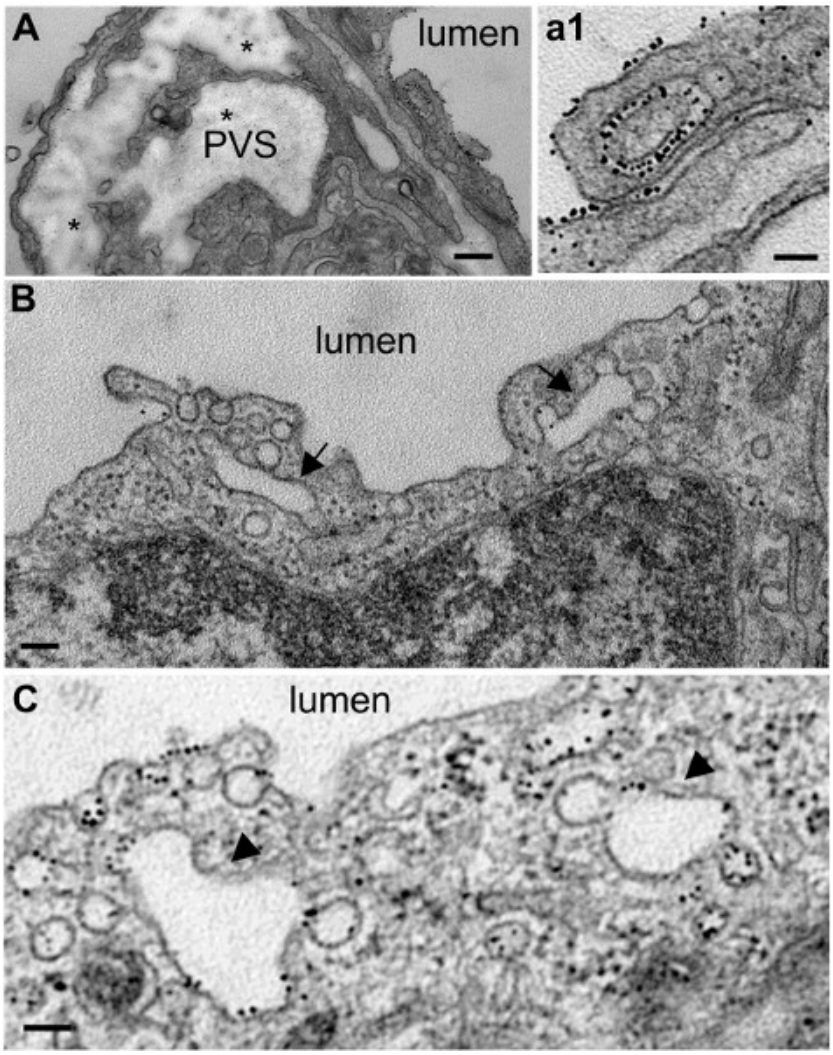

Figure 2. Acute perturbation of ITSN-1s expression induces pleomorphic endocytic /transcytotic intermediates. Representative EM images of membranous rings (**A, a1)**, tubular elements (**B,** arrows) and enlarged endosomes (**C,** arrowheads), loaded with 8 nm gold-albumin and associated with caveolae-like morphology. Note also the severe dilation of the perivascular space (pvs) and the proteinaceous edema (**A**). Bars: 250 nm (A); 200 nm (B) 100 nm (a1, C).

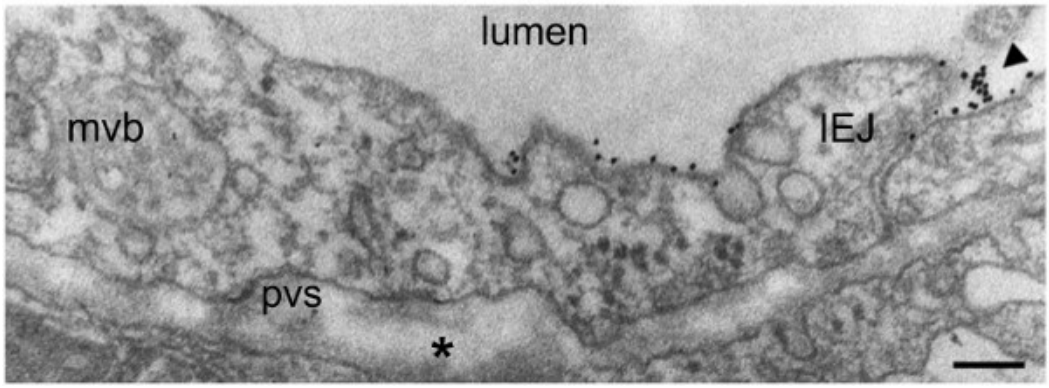

Figure 3. Chronic inhibition of ITSN-1s expression partially restores IEJ integrity. Filtration residues in the luminal introit of an IEJ (arrowhead). Mild dilation (*) of the pvs suggests leakiness of the IEJs. Multivesicular bodies (mvb), in close proximity of the plasma membrane have some of their internal small vesicles, labeled by 8 nm gold albumin particles. Bar: 100 nm.

Endocytic/transcytotic structures	Control	ITSN-1s siRNA, 72 hr	ITSN-1s siRNA, 24 d
Caveolae open to the lumen*	106.6 ± 9.5	50.46 ± 4.8	87.15 ± 5.9

Caveolae apparently free in the cytosol	173.5 ± 12.0	77.41 ± 6.3	143.0 ± 9.5
Total caveolae (luminal and free in the cytosol)	280.1 ± 21.5	127.86 ± 11.1	230.14 ± 15.4
Abnormal endocytic structures (enlarged endosomes)	2.65 ± .34	11.29 ± 2.7	14.93 ± 3.8
Caveolae clusters	3.95 ± .4	5.64 ±1.6	11.3 ± 2.5
Membranous rings	1.33 ± .04	9.47 ±2.4	12.8 ± 2.8
Tubules	.88 ± .05	4.0 ±1.3	18.84 ± 3.4

Table 1. Chronic inhibition of ITSN-1s expression causes the activation of alternative endocytic/transcytotic pathways and partially restores caveolae number. *Results are normalized per 100 µm EC length.

Discussion

Based on previous studies published by others[9] and us[1, 18] we developed this methodology for long term knock down of ITSN-1s *in vivo* by repeated intravenous administration (every 72 hr, for 24 consecutive days) of specific siRNA/liposome complexes. This experimental approach is efficient, can be used safely and repeatedly and it can be easily extended to study the involvement of a gene encoding any protein of interest in lung endothelium and pulmonary homeostasis. Under the experimental conditions set up by us for repeated siRNA/liposomes delivery, mice appeared normal with no signs of toxicity or immune responses. Systemic administration of $siRNA_{ITSN}$/liposome complexes is the most clinically relevant route for indications as cancer and other metabolic diseases. The lung is the first capillary bed encountered by the siRNA/liposome complexes injected intravenously and this may explain the efficient KD_{ITSN} in the lung.

One limitation of the study is the time dependent continuous aggregation of cationic liposomes[2]. To overcome it, the siRNA/liposome complexes were delivered shortly (less than 2 hr) after generation and preserved on ice at all times before use. Conventional cationic liposomes, such as the ones used in our study are proposed to be cleared by the reticulo-endothelial system. The rate of up-take by phagocytes can de decreased by liposomal pegylation. Steric stabilization with polyethylene glycol (PEG) coating prolongs liposomes' circulation time while having a more even tissular distribution. These factors become very important characteristics in anti-cancer therapies where prolonged circulation of the chemotherapeutic agents is desired[12].

In regard to the EM procedure, the minute amounts of tissue examined should be compensated for by systematic and extensive morphological and morphometric analysis as well as by examination of serial sections, when needed.

While our methodology optimizes the dosage schedule for sustained delivery and siRNA efficacy, further studies are needed to focus on the high efficiency identification of the right targets and on the assessment of risk/benefits parameters involved.

Disclosures

The authors have nothing to disclose.

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